

Lysis of Major Histocompatibility Complex Class I-bearing Cells in Borna Disease Virus-induced Degenerative Encephalopathy

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Summary

CD8⁺ as well as CD4⁺ T cells and macrophages are of crucial importance for the pathogenesis of Borna disease in rats. This virus-induced immunopathological disease of the brain is characterized by neurological symptoms in the acute phase and chronic debility associated with severe loss of brain tissue in the late stage. We demonstrate here the cytotoxic activity of T lymphocytes in the brain of intracerebrally infected rats. T cells isolated from the brain of infected rats lyse major histocompatibility complex (MHC) class I-bearing target cells in the absence of MHC class II. Borna disease virus (BDV)-infected syngeneic skin cells and astrocytes, the latter one of the relevant target cells *in vivo*, were significantly lysed whereas infected allogeneic target cells were not. Most relevant to the *in vivo* situation, primary brain cell cultures propagated from the hippocampus of BDV-infected rats containing considerable numbers of neurons were lysed *in vitro*. Blocking experiments using antibodies directed against MHC class I antigen provided further evidence for the presence and activity of classical cytotoxic T lymphocytes. Antibodies against MHC class II antigen did not influence lysis of skin target cells but had an effect on lysis of astrocytes at late time points. Lymphocytes isolated from spleen, peripheral blood, or lymph nodes did not show cytotoxic activity. These results verify, on the cellular level, earlier findings that strongly suggest the involvement of CD8⁺ T cells in brain cell lesions, resulting in brain atrophy long after infection of rats with BDV. This is further evidenced by the presence of CD8⁺ T cells in direct proximity to neuronal cell lesions. Interestingly, the cytolytic capacity, demonstrated *in vitro* and strongly correlated to organ destruction, does not result in elimination of the virus but the virus persists in the central nervous system.

In addition to horses and sheep as natural hosts, the experimental infection of individuals of various species with Borna disease virus (BDV),¹ a single-stranded RNA virus (1–3), usually results in an encephalomyelitis accompanied by severe neurological disorders (4–6). Recently, BDV-specific antibodies have also been detected in humans, indicating that humans might harbor a similar or identical agent that is possibly involved in psychiatric disorders (7–9). In the rat, the most intensively studied experimental animal, intracerebral or intranasal infection leads to a persistent, productive infection in the central nervous system (10–12) with virus replication in neurons, astrocytes, oligodendrocytes, and ependymal cells (10, 13–15).

By using newborn infected, athymic or immunosuppressed

animals, Borna disease (BD) was characterized as a virus-induced immunopathological disease (10, 16–19). Antiviral antibodies could be excluded from participating in the immunopathological process (10, 18), and the cell-mediated immune response itself was demonstrated to cause encephalitis and disease (reviewed in reference 20). The adoptive transfer of BDV-specific CD4⁺ T cells into tolerant, persistently infected rats inducing the full-blown disease proved the role of T helper cells in Borna disease (21, 22). Furthermore, information on the pathogenic importance of CD8⁺ T cells, which generally have never been successfully established as lines or clones from the rat, in BD was provided by use of immunomodulatory substances (18, 23) and T cell-specific mAb *in vivo* (24). These experiments revealed that CD8⁺ T cells are decisive in triggering a local delayed-type hypersensitivity reaction in the brain. Since CD8⁺ T cells have been shown to cause cytotoxicity in other virus models (reviewed in reference 25), we have undertaken experiments

¹ Abbreviation used in this paper: BDV, Borna disease virus.

to determine whether BDV-specific CD8⁺ T cells exert cytolytic activity in addition to their immunopathological function in BDV-induced encephalitis and disease. CTL dominate the initial cellular immune response after many virus infections and are effective in eliminating the virus from non-neural tissue. However, virus elimination from the brain may not be attained, as shown in the lymphocytic choriomeningitis virus model in mice (26). In the case of noncytopathic viruses, as demonstrated here by BDV, an efficient immune reaction is of considerable disadvantage for the host, which results in immune-mediated cell and tissue damage.

The studies presented here demonstrate the presence of MHC class I-restricted cytotoxic T cells in the brain but not in lymphoid organs of BDV-infected rats. The results support our view of the great biological importance of virus-specific CD8⁺ lymphocytes in Borna disease pathogenesis in general and for the destruction of virus-infected cells *in vivo*, in particular, resulting eventually in organ atrophy.

Materials and Methods

Experimental Animals and Virus. Lewis female rats obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany) were infected at an age of 5 wk by injection into the left brain hemisphere with 5×10^3 TCID₅₀ of BDV.

Infectivity Assay and Antigen Detection. Assays were essentially done as described previously (23). Briefly, virus infectivity from brain homogenates was determined on rabbit embryo brain indicator cells by indirect immunofluorescence using rat hyperimmune sera or BDV-specific mAb (27, 28). Virus-specific antigen was demonstrated in brain homogenates after SDS-gel electrophoresis by Western blotting. Detection of the BDV-specific 38/39- and 24-kD proteins was performed with polyclonal antibodies.

Induction and Detection of IFN. IFN- α/β was obtained from BDV-infected astrocyte cultures at various time points of cultivation. The presence of IFN was detected in conventional antiviral activity assays using vesicular stomatitis virus as described before (29). Briefly, serial twofold dilutions of test supernatants were prepared and added, together with the indicator cells (a permanent cell line from Lewis rat lens epithelium [L1; kindly provided by Dr. H. Rink, Institut für Strahlenbiologie, Bonn, Germany]), to flat-bottomed microtiter plates. Cultures were infected 24 h later with 100 ID₅₀ of VSV. After another 48 h supernatants were removed and cells were stained with crystal violet. Test supernatants were also dialyzed against glycine/HCl buffer (pH 2.0) overnight to exclude the presence of IFN- γ . A preparation from L1 cells obtained after infection with BDV was used as laboratory standard (29).

Isolation of Effector Cells. For cytotoxicity assays, lymphocytes from various tissues were obtained. Lymphocytes from peripheral blood, spleens, and lymph nodes were purified on a Lymphocyte-R gradient (Cedarlane Laboratories, Hornby, Canada) according to the manufacturer's specifications. Lymphocytes from the brain were isolated by a method previously described by Irani and Griffin (30). The animals were anesthetized with ketaminehydrochloride and perfused with balanced salt solution (BSS). The brain was homogenized carefully through a stainless steel mesh and collected in BSS containing Collagenase D (0.05%), trypsin inhibitor (TLCK; 0.1 μ g/ml), DNase I (10 μ g/ml), and Hepes (10 mM). The cell suspension was stirred at room temperature for 1 h and allowed to settle for 30 min. The supernatant was pelleted at 200 g for

5 min and resuspended in 10 ml Ca/Mg-free PBS. 5 ml of the suspension was layered on top of 10 ml of a modified RPMI/Ficoll gradient and centrifuged at 500 g for 30 min. The pellet containing the lymphocytes was resuspended in IMDM with 2% FCS, and the cells were counted for further use.

Propagation of BDV-infected Neurons *In Vitro*. Tissue specimens of 1 mm³ were taken from the hippocampus of rats 14 d after BDV infection and propagated in RPMI containing 15% FCS, nerve growth factor (10 ng/ml), and Matrigel (50 μ g/ml) (Becton Dickinson & Co., Heidelberg, Germany) in 24-well microtiter plates. After 4 h, nonadherent tissue particles were removed and control wells were stained for typing and viability of the adherent cells. The percentage of neurons was then calculated by visual counting and the cells were immediately used for the cytotoxicity assay.

Target Cells and MHC Expression. The following target cells were used: (a) BDV-infected (BDV-F10) and uninfected (NL-F10) histocompatible astrocytes (the homogeneous astrocytic cell line F10, cloned from a primary Lewis [LEW] astrocyte culture by limiting dilution, was provided by Dr. H. Wekerle, Munich, Germany; 31); (b) compatible and incompatible skin cell cultures; and (c) primary neuronal cells. Skin cell cultures were obtained from 2-wk-old Brown Norway (BN) rats, which are histoincompatible both at the RT1 (MHC I) and RT2 (MHC II) locus, and from syngeneic LEW rats. These cultures were designated BDV-BN, NL-BN (not infected), BDV-LEW, and NL-LEW (not infected). On all cells the expression of MHC antigen using mAbs OX-18 (class I) and OX-6 (class II) was examined by indirect immunofluorescence.

***In Vitro* Treatment of Lymphocytes with Antibody Plus Complement.** Purified lymphocytes were treated at a concentration of 2×10^6 with a polyclonal rabbit anti-asialo GM1 antibody (1:300; Wako Chemicals, Düsseldorf, Germany) plus complement *in vitro*. Cells were incubated with antibodies for 45 min on ice, washed twice, and then incubated with 1 ml of a 1:12 dilution of low-tox rabbit complement for rat lymphocytes (Cedarlane Laboratories) for 30 min at 37°C.

***In Vitro* Cell-mediated Cytotoxicity.** Aliquots of 10^7 target cells were labeled with 0.2 mCi ⁵¹Cr at 37°C for 1 h and washed three times with medium. The labeled target cells were cocultured with effector cells from BDV-infected rats at E/T ratios of 30:1 for brain lymphocytes and 100:1 for all other effector lymphocytes, and threefold dilutions of the original concentration in a final volume of 200 μ l/well. Some tests were performed in the presence of mAb directed against MHC class I or II determinants. Furthermore, adherent cells were removed by incubating effector cell populations on plastic dishes for 30 min at 37°C. After various periods of time (6–18 h), 50- μ l samples were collected and counted in a gamma counter. The percentage of ⁵¹Cr release was calculated according to the formula: $100 \times (\text{test release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$, where test release is in the presence of effector cells, spontaneous release is in the presence of medium alone, and maximal release is by 1 N HCl. Spontaneous release never exceeded 25%. Cytotoxicity of brain lymphocytes from BDV-infected rats against BDV-infected hippocampal cells propagated *ex vivo* was determined 6 h after cocultivation. After removing the medium, the plates were washed in PBS and the adherent cells were fixed and stained with cresyl violet or with neurofilament-specific antibodies to identify neurons. The cytotoxicity was then calculated by visual counting of the vital neurons in the test wells in comparison with wells containing neurons from uninfected rats and with wells containing BDV-infected neurons that were covered with anti-MHC class I antibody OX-18 1 h before adding the effector cells.

Immunofluorescence and Antibodies to Cell Determinants. To test cells for the expression of BDV and MHC antigen, the indirect immunofluorescence technique was used. Cells were fixed with acetone for 20 min and for 10 min in chloroform at room temperature. Nonspecific staining was blocked by incubation with undiluted porcine serum for 30 min. After several washings with PBS the primary antibodies were incubated for 60 min at room temperature: mAb OX-18 (MHC class I, 1 mg/ml; 1:100), mAb OX-6 (MHC class II, 1 mg/ml; 1:100), mAb 38/15H7 (BDV; 1:100), mAb NR4 (neurofilament, 68 kD; 1:100). Surface expression of MHC antigens was determined on BDV-infected and uninfected cells without fixation in the presence of sodium azide in the cold. After several washes FITC-labeled secondary antibodies (Sebak, Aidenbach, Germany) were added for 60 min at 4°C at concentrations of 1:50. The slides were finally mounted with PBS/antifade solution. Double immunofluorescent staining of neurofilaments and MHC class I antigens was performed by use of the avidin-biotin method with FITC for the anti-neurofilament (NF) 68-kD mAb NR4 and a Texas red-coupled anti-mouse IgG for visualization of OX-18.

Anti-T Cell Treatment of Rats. BDV-infected rats were treated with mAbs directed against the CD8 (OX-8) or CD4 molecule (W3/25) as reported earlier (20).

Histology. Brain tissue was removed immediately after the animals were killed and either frozen in isopentane at -150°C or fixed in buffered formalin. Sections were stained with hematoxylin and eosin of Nissl stain. Reduction of brain tissue in BDV-infected rats was calculated as follows: mean diameter of the dorsal cerebral cortex of all animals in a group (BDV-infected and either untreated

or treated in vivo with the CD8-specific mAb OX-8 or the CD4-specific mAb W3/25). Measurement was performed in three different frontal sections according to the rostral-caudal coordinates corresponding to 0-0.6, 2.6-3.6, and 4.6-5.6 of the coordinate system of de Groot (32).

Results

Antigen Expression by Target Cells. All BDV-infected cell cultures were shown to express BDV-specific antigens, as detected by indirect immunofluorescence using polyclonal rat sera or mouse mAb (Fig. 1 A). Furthermore, the expression of MHC antigens in BDV-infected and uninfected cells was tested. Both astrocyte and skin cell cultures, irrespective of whether infected or not, spontaneously expressed MHC class I antigen as detected by mAb OX-18 (Fig. 1 B). In contrast, neither skin cells (Fig. 1 C) nor astrocytes expressed detectable amounts of MHC class II antigen spontaneously, the latter results confirming our previous findings (31). Distinct Ia antigen expression could only be induced by pretreatment with rIFN- γ (20 U/ml) on astrocytes (31) but not on skin cell cultures, as shown by using the MHC class II-specific mAb OX-6 (Fig. 1 D). Thus, BDV-infected cells used as target cells in cytotoxicity assays only expressed MHC class I antigen. Additionally, we included experiments to determine whether infected astrocytes can produce IFN in vitro, which might result in an upregulation of MHC antigens after

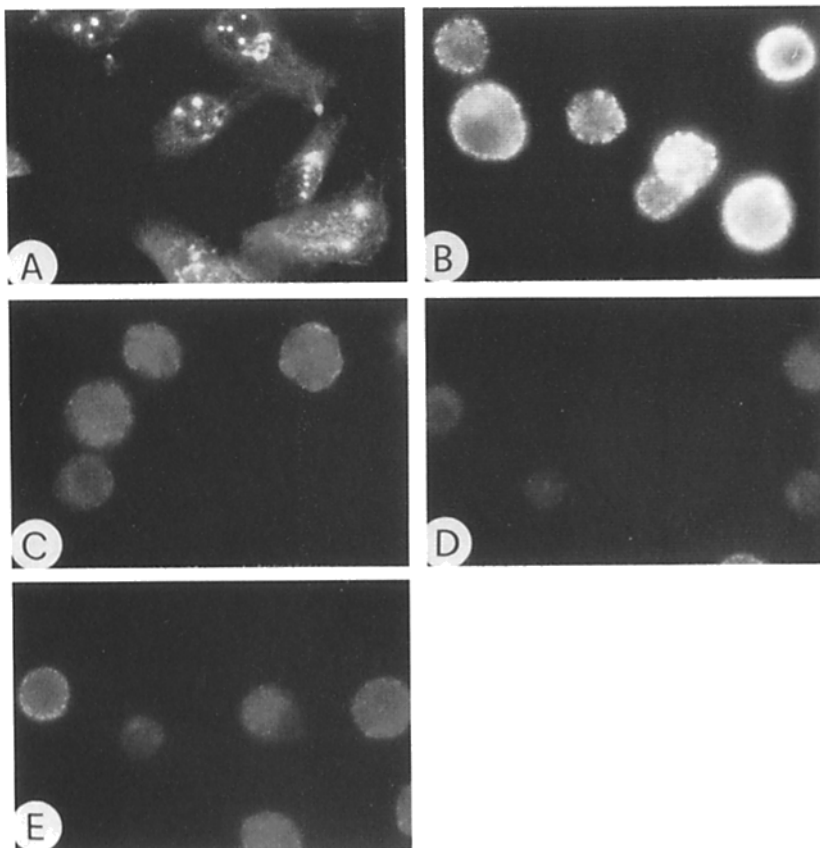


Figure 1. BDV antigen and MHC antigen expression on target cells. Presence of BDV-specific antigen (A, BDV-specific mAb) and MHC class I antigen (B, mAb OX-18), absence of MHC class II antigen without further treatment (C, mAb OX-6) and after treatment with rIFN- γ (100 U/ml; D, mAb OX-6), and control for FITC-labeling without primary antibody (E) on infected LEW skin fibroblasts.

prolonged incubation periods during cytotoxicity assays. Testing of supernatants from BDV astrocytes revealed no antiviral activity at 12 h, whereas maximally 100–220 U was found in BDV-F10 supernatants beyond this time point (Fig. 2). After 48 h of incubation, cells tested for expression of MHC antigens revealed the presence of class I but not class II antigens. Since treatment of supernatants at pH 2 did not result in the reduction of antiviral activity (data not shown), these results argue for the presence of IFN- α/β and the absence of IFN- γ , which might have induced MHC class II antigen in target cell cultures.

Presence of Cytotoxic T Cell Activity in Lymphocyte Preparations from the Brain. Testing of lymphocytes isolated from the brain of BDV-infected rats between days 10 and 21 postinfection revealed differences in their capacity to lyse virus-infected target cells. Whereas only very few brain lymphocytes without lytic activity were present at day 10 postinfection, high lysis was found beyond day 14, in parallel with or preceding the onset of clinical symptoms for 1 d (data not shown). Since the largest numbers of lymphocytes could be isolated on day 21 postinfection, this time point was chosen for all subsequently described experiments. Day 21 BDV-immune brain lymphocytes were coincubated with infected (BDV-LEW) and uninfected (LEW) skin cultures (Fig. 3, A and B). After 6 h of coincubation, moderate cytotoxic activity could be detected ranging from 20 to 40% in five independent experiments; at 9 h, high lysis (45–70%) of BDV-LEW was found whereas uninfected target cells were not lysed. As relevant

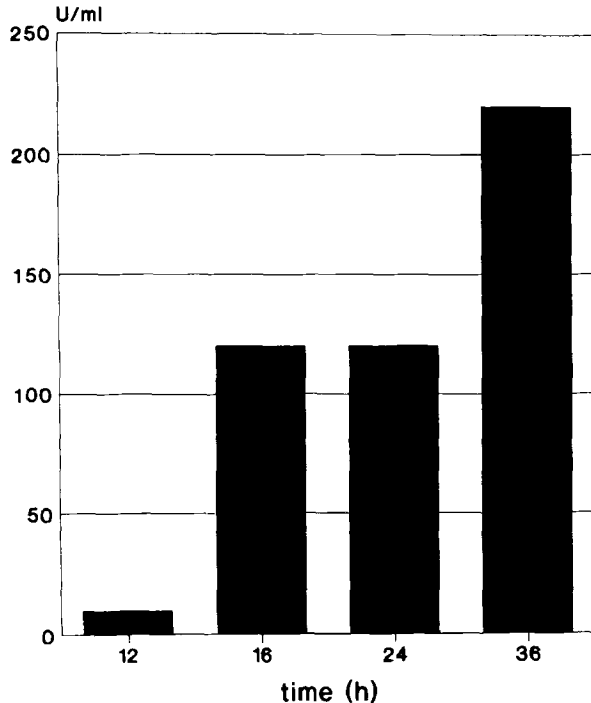


Figure 2. Antiviral activity in supernatants from BDV-infected rat astrocytes. Supernatants of cultures were titrated on rat lens epithelial cells for protection against the cytopathic effect of vesicular stomatitis virus. Titers given are units of IFN found to protect 50% of indicator cells.

target cells to the *in vivo* situation, BDV-infected astrocytes were also tested in cytotoxicity assays. It was found that lysis of astrocytes was insignificant at 6 h, increased until 9 h to significant values, and cytotoxicity reached a maximum between 12 and 18 h (Fig. 3, C and D). These kinetics were highly reproducible for both types of target cells used.

Absence of Cytotoxic Activity from Lymphoid Organs. After the presence of cytotoxic cells had been established in the brain, it was of interest to determine whether cytotoxic activity could also be found in lymphocyte preparations from other lymphoid tissue. Therefore, lymphocytes were purified from spleen, peripheral blood, and abdominal lymph nodes and compared with lymphocytes isolated from the brains of the same animals in cytotoxicity assays. Cytotoxic activity could not be detected in lymphocytes from BDV-infected rats at day 21 postinfection apart from brain lymphocytes (Fig. 4). Additional testing of lymphocytes isolated from the blood of infected rats at earlier time points surprisingly revealed the same results (data not shown).

BDV-specific Brain Effector Cells Are MHC Restricted. After the cytotoxic activity of brain lymphocytes had been established on MHC class I-bearing skin cell cultures, experiments were performed to prove MHC restriction. Therefore, cytotoxicity assays were performed on compatible (BDV-LEW) and incompatible (BDV-BN) skin fibroblast target cells. Syngeneic skin cells were clearly lysed whereas incompatible BDV-infected skin cells from BN rats were not (Fig. 5 A). Both types of skin target cells were from persistently BDV-infected cultures in which 100% of the cells were found positive for virus-specific antigen by immunofluorescence or Western blot analysis with BDV-specific rat antisera or mAbs. Virus titers obtained after freezing and thawing of all infected cells were fully comparable (data not shown). Depletion of adherent cells from the effector cell population did not result in a different reaction pattern, and the possible presence of NK cells in effector cell populations was excluded by cytotoxicity assays on NK-sensitive YAC-1 target cells. (Fig. 5 B).

Treatment of effector lymphocytes with anti-asialo GM1 antibody, a common marker of NK cells and of the majority of CD8⁺ T cells in the rat, resulted in a complete absence of lysis (Fig. 5 C). Furthermore, blocking experiments with mAb OX-18 (anti-MHC class I) were performed. Lysis of syngeneic skin target cells was completely inhibited by anti-MHC class I antibodies (Fig. 5 D). Since lysis on astrocytes, which had been shown to be capable of expressing MHC class II antigen, had increased at time points later than 9 h, possibly due to IFN- γ induction during the coincubation of effector lymphocytes and target cells, we also tested whether anti-MHC class II had any influence on the lysis of infected astrocytes. The presence of anti-MHC class I antibody again resulted in a significant decrease (75–85%) of cytotoxicity at 9 h. However, some reduction in lysis (20–35%) was also found of anti-MHC class II antibody, whereas an irrelevant isotype-matched influenza nucleoprotein-specific mAb did not reduce lysis (Fig. 5 E).

Lysis of BDV-infected Brain Cells In Vitro Primary brain cell cultures obtained from BDV-infected rats were also used

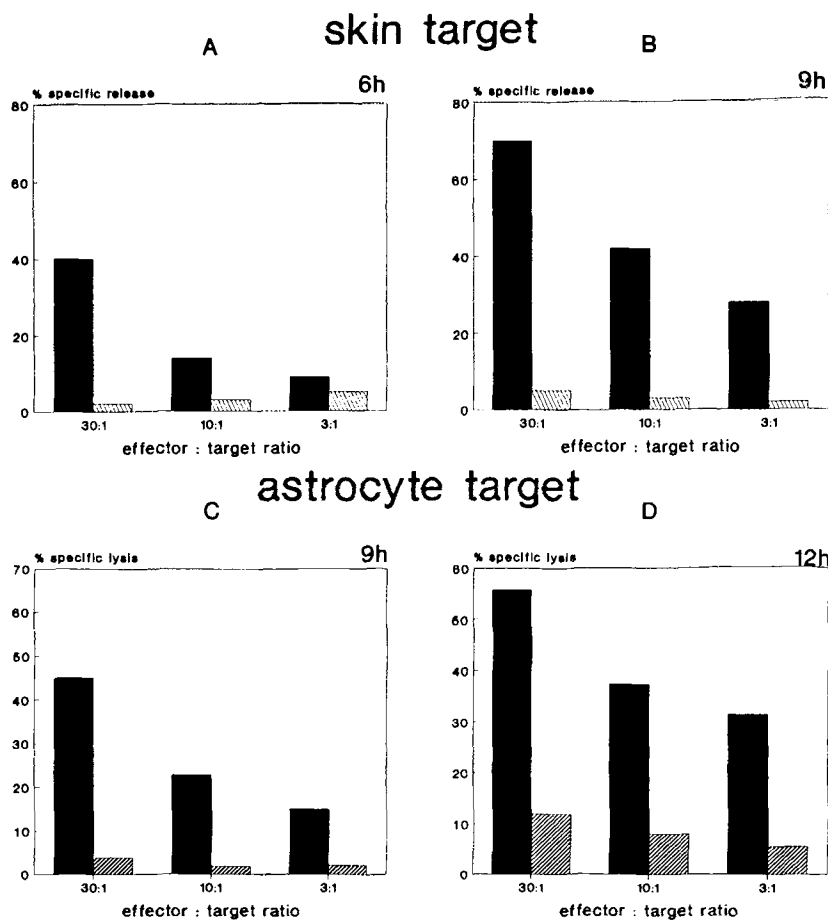


Figure 3. Lysis of histocompatible target cells by lymphocytes isolated from the brain of BDV-infected rats. Kinetics of cytotoxicity on skin fibroblasts at 6 (A) and 9 h (B), and on astrocytes at 9 (C) and 12 h (D).

as target cells in cytotoxicity assays. These cultured cells were characterized in vitro to contain numerous neurons, as shown by immunofluorescence with a neurofilament-specific mAb, and express MHC class I antigen (Fig. 6). However, since primary neuronal cultures are known to be particularly sensitive to manipulations, e.g., ^{51}Cr labeling, viability of neurofilament-containing cells, i.e., neurons, after cocultivation with effector cells was determined. As can be seen from Fig. 7 neurons as relevant target cells to the in vivo situation were significantly lysed in vitro during a 6-h cytotoxicity assay.

Demonstration of Brain Cell Lesions In Vivo and Effect of Anti-T Cell Antibodies. To correlate the in vitro findings on cytotoxic T cell activities with the in vivo situation, histological examination of brains from BDV-infected rats was performed. Simultaneously with the appearance of inflammatory reactions, first neuronal degenerations were found in the brain (Fig. 8 A). Additionally, severe astrocytic and perivascular edema was seen (Fig. 8 B). Brain cell lesions occurred in direct proximity of CD8^+ cells (Fig. 8 C). The severeness of brain destruction in BDV-infected rats not treated with CD8 -specific mAb is shown (Fig. 9 A) at day 50 postinfection. Adult BDV-infected animals show a reduction of brain tissue of $\sim 60\%$ as compared with uninfected rats (Fig. 10). In contrast, no inflammatory reaction (24) and little brain tissue

loss were found in rats treated with antibody OX-8 directed against CD8^+ T cells (Fig. 9 B), whereas anti- CD4 -treated BDV-infected rats still showed significant loss of brain substance (Fig. 10). In all rats, however, virus-specific antigen as detected by Western blot analysis and immunohistochemistry on the one hand and in situ hybridization (data not shown), and infectious virus as shown in infectivity assays on the other hand, persisted in the brain (Fig. 10). The virus titer on the per gram basis was not different in BDV-infected rats showing severe brain atrophy than in BDV-infected and OX-8-treated rats without tissue degeneration.

Discussion

Lymphocytes isolated from the brain of rats infected with BDV exert cytolytic activity on BDV-infected target cells in a MHC class I-restricted manner. Effectors exhibited cytotoxic activity on primary neuronal cells cultivated from BDV-infected syngeneic Lewis rats and lysed syngeneic infected skin cells but did not exert cytotoxicity on allogeneic MHC-mismatched skin cells obtained from BN rats. In addition to MHC restriction, experiments using mAb to block the recognition of MHC molecules on target cells clearly showed

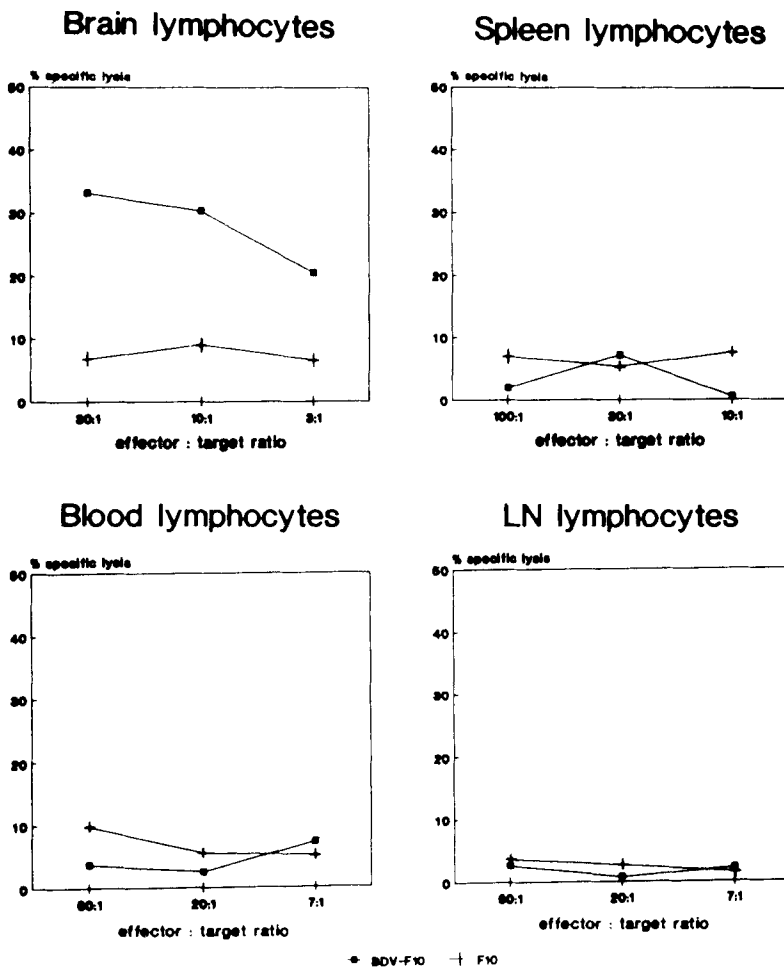


Figure 4. Presence of cytotoxic activity in lymphocyte preparations from various tissues. Lymphocytes were isolated from the indicated tissues by Ficoll gradients (Lympholyte-R) and tested in a 9-h assay. Note the different E/T ratios.

that lysis was mediated by classical MHC class I-restricted cytotoxic T cells.

We previously showed that CD8⁺ T cells are of pathogenic importance in BD and contribute most to the acute immunopathological disease, particularly shown by the increase of CD8⁺ cell numbers in parallel with the onset of the disease (23, 24). In addition to acute Borna disease there are drastic chronic affects on the central nervous system (33). A severe hydrocephalus internus can be found in rats infected with BDV. This hydrocephalus does not result secondarily from hydrocephalus occlusus (34) or from hypoxia (our unpublished observation). As morphologic parameter of the process leading to the destruction of brain tissue, necrosis of brain cells, as represented by severe astrocytic and perivascular edema, as well as nerve cell degenerations have been seen (Fig. 8). Similar pathological changes in the brain tissue, suggested to be due to immune-mediated lysis, were observed earlier (10); however, no direct evidence for immunopathological destruction or the cell responsible for the loss of brain tissue has yet been provided (10, 13, 14, 35).

The prime immune response to eliminate virus from the host is the generation of CTL that recognize virus-specific

determinants together with the class I MHC, a glycoprotein expressed on the surface of most host cells in the body (36, 37).

In this study, we could demonstrate the cytolytic activity of MHC class I-restricted lymphocytes isolated from the brain of infected animals. The lysis exerted by BDV-specific rat lymphocytes was delayed and therefore the kinetics were different from mouse cytotoxicity assays that only take maximally 4–6 h. However, rat CTL specific for vaccinia virus or directed against tumor determinants also show protracted lytic activity in vitro (38, 39). Antibody-mediated cytotoxicity operative in BD can be excluded for several reasons: first, treatment of BDV-infected rats with suboptimal doses of cyclosporine A or anti-CD4 mAb treatment resulted in the inhibition of virus-specific antibody synthesis but could not inhibit encephalitic lesions and disease (18, 24); second, pathological changes in the brain can be observed before antibodies are detectable; and third, athymic rats synthesize virus-specific antibodies but do not show encephalitis (17). NK cell activity could be excluded experimentally in vitro by assays using the NK-sensitive target cell YAC-1; furthermore, astrocytes (40) and occasionally fibroblasts (41), which were used in this study, do not serve as NK targets. In lack of a complement-binding

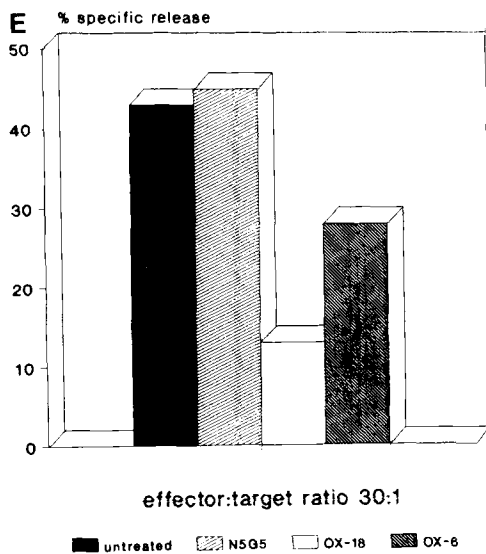
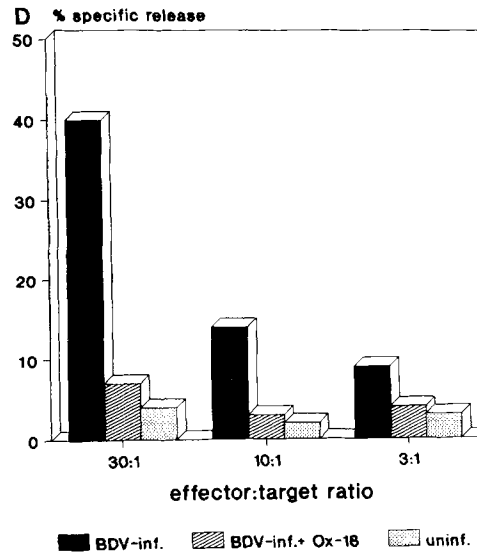
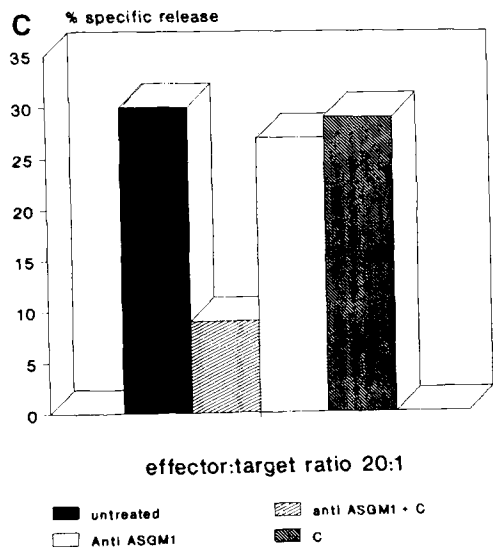
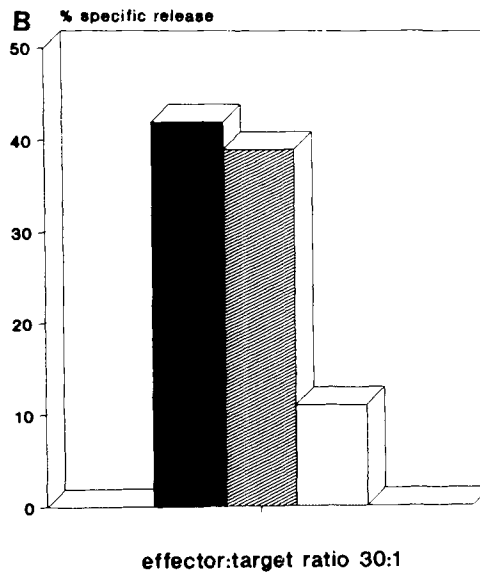
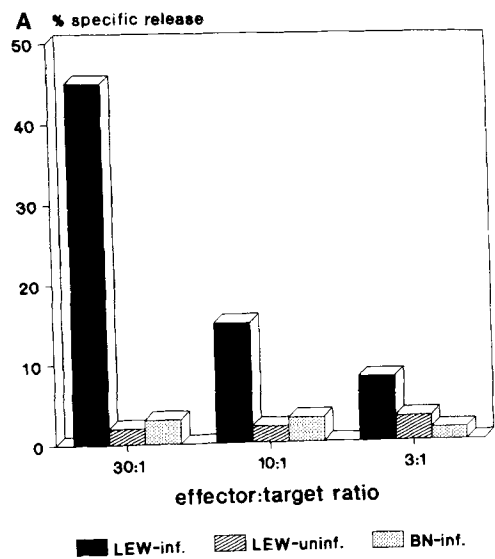


Figure 5. MHC restriction of CTL isolated from the brain of BDV-infected rats. (A) Absence of lysis on allogeneic MHC-mismatched skin fibroblast targets from BN rats. (B) Lysis of BDV-infected target cells without further treatment of brain effector cells (■) and after depletion of macrophages (▨); absence of significant NK activity on YAC-1 target cells (□). (C) Loss of lytic activity of brain lymphocytes on infected syngeneic skin fibroblasts after treatment with antibodies to the NK/CD8⁺ T cell marker asialo GM1. (D) Inhibition of lysis in the presence of anti-MHC class I antibody (mAb OX-18) on LEW skin fibroblasts. (E) Effect of antibodies against MHC class I and II (mAbs OX-18 and OX-6) and an irrelevant isotype-matched mAb (N5G5) on lysis of BDV-infected astrocytes.

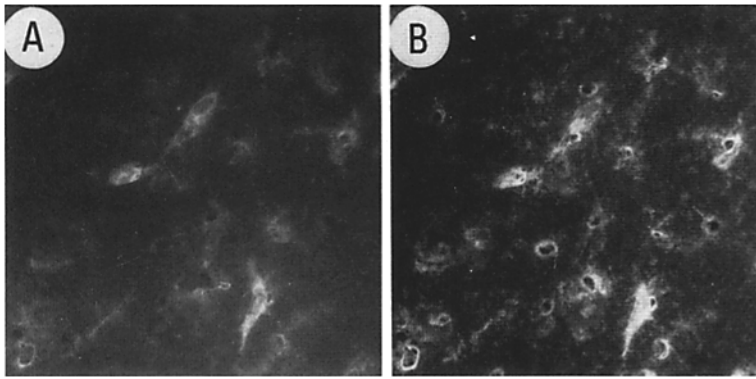


Figure 6. Expression of MHC class I antigen on BDV-infected hippocampus neurons propagated in vitro from rats 14 d postinfection for brain lymphocyte-mediated cytotoxicity assay. Immunofluorescence double staining with an anti-neurofilament mAb (A) and anti-MHC class I mAb OX-18 (B). Presence of BDV antigen was tested in parallel staining (not shown) ($\times 240$).

mAb specific for rat CD8⁺ cells, the T lymphocyte phenotype of cytotoxic cells was confirmed using an anti-asialo GM1 antibody that was able to fix complement. The asialo GM1 marker is expressed on NK cells and the majority of CD8⁺ T cells in the rat (42). Antibodies directed against asialo GM1 can readily abrogate cytotoxic activity of virus-specific T cells (43). Treatment of effector cells with the antibody completely abrogated lysis of target cells, confirming that CTL are operative in MHC-restricted lysis of BDV-infected target cells.

Despite the activity of cytotoxic cells, BDV persists in the brain. This phenomenon remains to be investigated more thoroughly. This finding is not unique since a similar situation has been reported for lymphocytic choriomeningitis virus infection in mice, in which virus clearance is not accomplished despite the presence of cytotoxic T cell activity (26). It can be envisaged that in vivo the cytotoxic T cell response might

occur too late with regard to a limitation of virus replication at an early stage of infection, since the virus spreads readily and quickly throughout the brain.

Interestingly, no cytotoxic T cell activity was found in peripheral lymphoid tissue preparations 21 d postinfection. Additional testing of lymphocytes purified from the peripheral blood of infected rats between days 5 and 15 also failed to reveal antigen-specific cytotoxic activity. A similar observation has been reported for hepatitis C virus infection in humans, where no virus-specific cytotoxic T cell activity was detectable in PBL from patients in which hepatitis C virus-specific cytotoxic cells were found among liver-infiltrating lymphocytes (44). In BD, this finding might be attributable to a low frequency of BDV-specific cytotoxic T cells in the periphery and the subsequent and selective homing of activated cells from the periphery to the site of antigen presentation and tissue damage. However, we cannot exclude that CD8⁺ T cells also might be directly stimulated locally, e.g., by dendritic cells and astrocytes (45–48). It is of note in this respect that BDV antigen is present only in the nervous system of adult infected rats and virus does not measurably replicate outside the nervous system (49). However, it is also known that intracerebral infection results in the presence of virus in the periphery (50), and we have provided evidence for an early peripheral priming of virus-specific T cells in BD (18).

MHC class I antigen has been demonstrated in the brain of BDV-infected rats (15, 23). Here we show the presence of MHC class I antigen on neuronal cultures ex vivo. Although, under normal conditions, the nervous system and especially neural cells have low levels of MHC antigen, greater expression occurs during pathologic situations (51, 52). This notion is supported by observations that IFN- γ induces MHC class I antigen expression on cells of neuronal origin (53, 54). Whereas no conspicuous class II antigen expression was found on neurons in BD, astrocytes also expressed MHC class II antigen. Taking this in vivo finding into account, we investigated which MHC antigens were expressed on the surface of the target cells used in this study. In addition to skin fibroblast we performed cytotoxicity assays on targets relevant to the in vivo situation, namely on neuronal and astrocyte cultures persistently infected with BDV. Whereas MHC class II antigen could not be detected on fibroblasts in the present

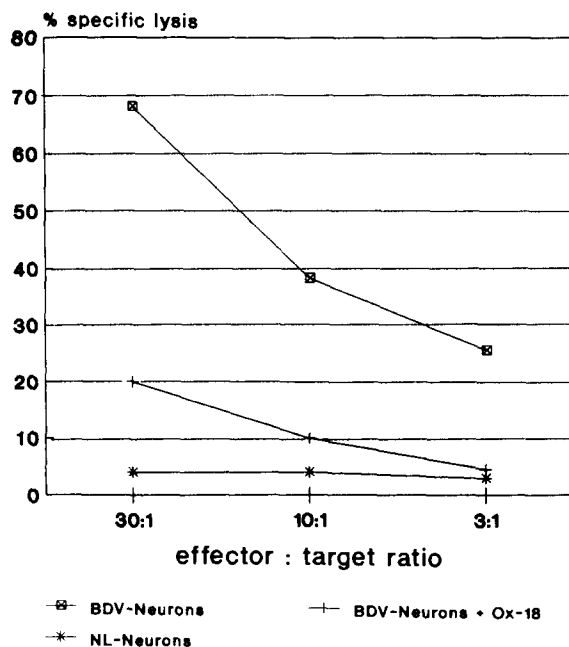


Figure 7. Lysis of primary neuronal cell cultures by BDV effector lymphocytes (6-h assay).

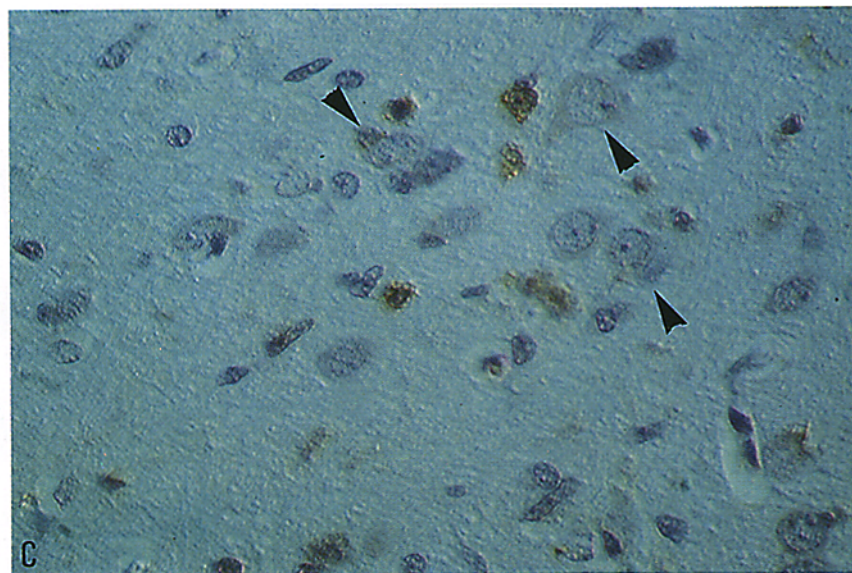
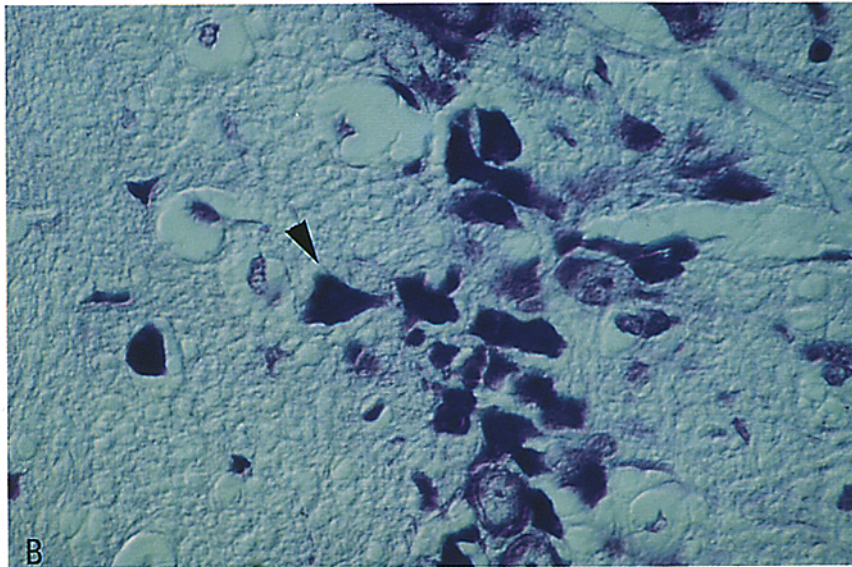
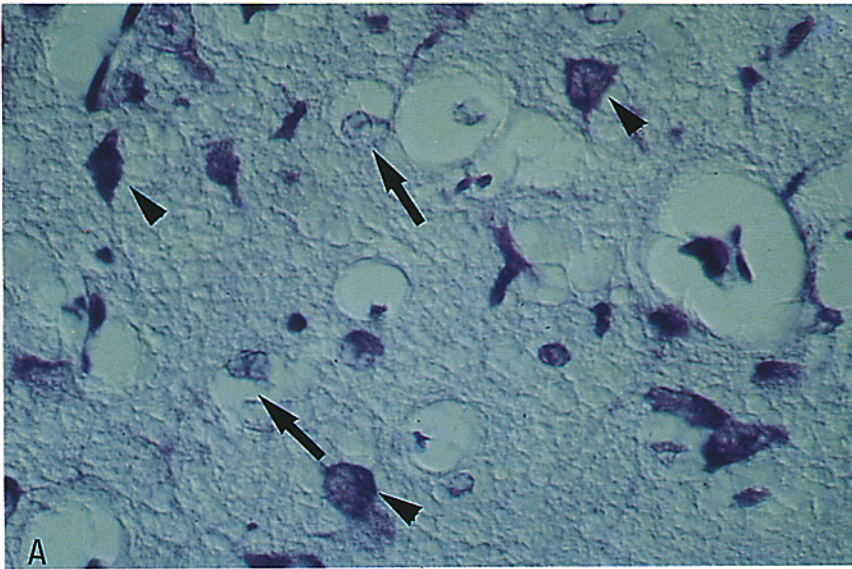


Figure 8. Pathological lesions to the brain of BDV-infected rats. Severe astrocytic and perivascular edema (A) as well as nerve cell degenerations (B) in the cortex and the hippocampus formation at day 14 postinfection. Note the direct proximity of immunostained CD8⁺ cells to the nerve cells (C). *Arrows*, astrocytes; *arrowheads*, neurons. (A and B) Nissl staining; (C) ABC-immunoperoxidase staining with mAb Ox-8, counterstained with hemalaun.

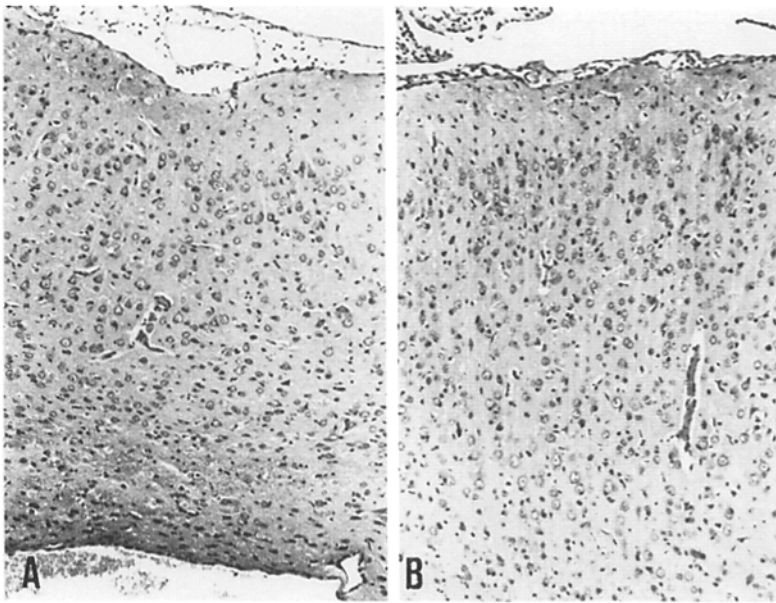


Figure 9. Brain cell atrophy in BDV-infected rats. (A) Severe brain atrophy is observed in an adult untreated LEW rat 50 d postinfection with a "burned-out" inflammation. (B) The brain of a BDV-infected rat treated with mAb OX-8 in vivo without atrophy and signs of inflammation. Hematoxylin-eosin staining; $\times 80$.

study or in a previous report (55), and neurons did not show conspicuous MHC class II antigen expression in vivo (Bilzer, T., and L. Stitz, manuscript in preparation), astrocytes expressed MHC class II antigen only after induction with IFN- γ (31, 56). IFN produced by BDV-infected astrocytes did not

induce Ia antigen expression on indicator cells. This result agrees with the finding that IFN- α/β , and in particular IFN produced by astrocytes, upregulates MHC class I but not class II expression (57, 58). Furthermore, blocking experiments with anti-MHC class I antibodies demonstrated the restriction specificity of the cytotoxic T cells. However, the fact that blocking was not complete and cytotoxicity could be found after prolonged periods of time (12 h) on astrocytes, indicating the characteristic kinetics of lysis from MHC class II-restricted T cells, Ia-restricted CTL might be present in the brain of BDV-infected rats. This interpretation agrees with the reported cytotoxic activity of a BDV-specific CD4⁺ T cell line (31). These virus-specific T cells might participate in the pathogenic process of late changes by direct action on MHC class II-bearing astrocytes, which indirectly may result in effects on neurons. For formal reasons we therefore cannot exclude that CD4⁺ T cells might contribute to the destruction of brain tissue. However, there is no significant MHC class II antigen expression on neurons in BDV-infected rats, whereas various other cell types in the brain express high levels of this antigen (Bilzer, T., and L. Stitz, in preparation) and treatment with mAb directed against CD4⁺ T cells still resulted in a loss of brain tissue of >50% vs. only 10% in anti-CD8-treated BDV-infected rats. Therefore we conclude that the impact of CD4⁺ T cells may be little. The very limited brain cell degeneration in anti-CD8-treated rats might reflect some interference of BDV with the cellular metabolism, as suggested recently by Carbone et al. (12). Wong et al. (54), and more recently Joly et al. (59), have discussed the possibility that neuronal cells evade immunopathological destruction by lack of MHC class I expression. The findings here suggest that this pathway for neurons to escape cellular defense mechanisms may not be generalized.

In conclusion, the activity of CD8⁺ T cells obviously is not only important in the early phase of BD, where it ap-

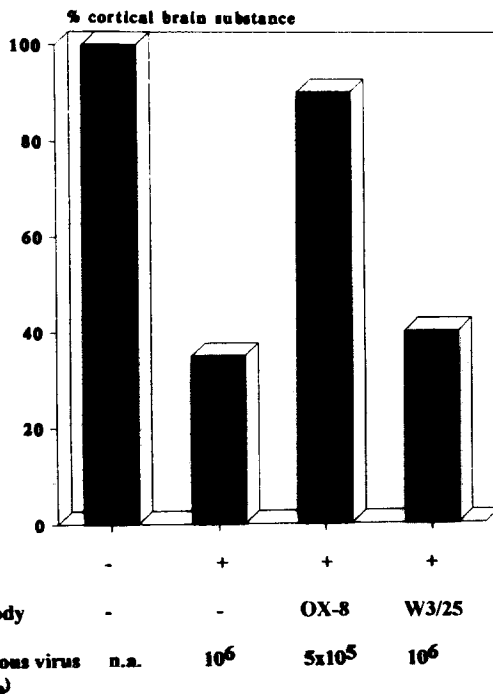


Figure 10. Reduction of brain substance in BDV-infected rats with and without anti-T cell antibody treatment at day 70 postinfection (OX-8, anti-CD8; W3/25, anti-CD4). For calculation of tissue reduction see Materials and Methods. Virus titers are given on the per gram tissue basis determined by end-point titrations.

pears to be decisive in triggering the local CD4⁺ T cell-mediated delayed-type hypersensitivity reaction, but also apparently predominates in the mechanisms of the destruction of brain cells, leading to atrophy and clinically to dementia and chronic debility. Therefore, BD in rats provides an in-

teresting model to study immunopathological mechanisms active during persistent virus infections and also appears to be a unique model to investigate T cell-mediated tissue destruction in vivo.

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